GENOME-WIDE ASSOCIATION STUDIES

Towards identifying genes underlying ecologically relevant traits in *Arabidopsis thaliana*

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Abstract | A major challenge in evolutionary biology and plant breeding is to identify the genetic basis of complex quantitative traits, including those that contribute to adaptive variation. Here we review the development of new methods and resources to fine-map intraspecific genetic variation that underlies natural phenotypic variation in plants. In particular, the analysis of 107 quantitative traits reported in the first genome-wide association mapping study in *Arabidopsis thaliana* sets the stage for an exciting time in our understanding of plant adaptation. We also argue for the need to place phenotype–genotype association studies in an ecological context if one is to predict the evolutionary trajectories of plant species.

Adaptive walk
The evolutionary path taken by a population towards a new phenotypic optimum; it is defined by the number, phenotypic size and temporal sequence of genetic changes.

Life history
Life history traits are closely related to fitness traits, such as number and size of offspring, age at first reproduction, and reproductive lifespan and ageing.

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Details regarding how adaptation proceeds remain elusive. From a theoretical perspective, several questions have been addressed[1–6]. How many genes are expected to be involved in a specific adaptation? Does the origin of adaptation (new mutations versus standing genetic variation) affect the adaptive walk to a new phenotypic optimum? What is the distribution of phenotypic effects that are fixed during an adaptive walk? Unfortunately, as in other long-standing debates in evolutionary ecology, arguments can flourish in the absence of data. To fill the gap between theory and data, an important goal is to identify the genetic basis of adaptive trait variation.

In plants, the identification of genes that underlie phenotypic variation can have enormous practical implications by providing a means to increase crop yield and quality in an agricultural context[7]. At the same time, the identification of ecologically important genes should help in predicting the evolutionary trajectories of plant populations[3,8–10]. *Arabidopsis thaliana* is a convenient species for these pursuits because it has a worldwide distribution and, as such, encounters diverse ecological conditions[9,11–14], leading to adaptive variation for many morphology, life history and other fitness-related traits[15]. During the past two decades, molecular tools have been developed to assist in the mapping of quantitative trait loci (QTLs) in experimental populations, but these tools remain laborious[16]. Recently, the first study of genome-wide association (GWA) mapping in plants was reported[17], bringing a breath of fresh air to the area of gene discovery. The high resolution conferred by GWA mapping facilitated mapping of the genetic bases of 107 diverse phenotypes, including flowering time, pathogen resistance, seed dormancy, ionomics and vegetative growth. Long considered the privilege of human mapping studies, GWA mapping has now emerged as a powerful alternative approach to finely dissect the intraspecific genetic variation that underlies phenotypic variation in plants[18–20].

Here we describe the connections among long-established strategies (such as traditional linkage mapping), recently developed approaches (such as GWA mapping) and upcoming methods (such as nested association mapping (NAM)) for finely mapping QTLs underlying natural variation. We review several powerful GWA mapping approaches and analytical methods that have been developed, as well as the available genotypic and phenotypic resources that are linked to the approaches. Because genetic variation is exposed to natural selection in contrasting ecological habitats, we emphasize the importance of ecological context. First, the spatial and temporal scale at which selection acts will determine the appropriate populations for GWA mapping[21]. Second, the cues perceived by a plant are far more complex, and not well captured, by simple growth-chamber conditions. This highlights the need to measure phenotypes in realistic conditions[22,23]. Third, the heterogeneity of the habitats encountered by *A. thaliana* suggests that experiments designed to phenotype plants in multiple locations will provide more robust results than

Table 1 | **Advantages and drawbacks of methods for identifying the genetic basis of complex traits in *Arabidopsis thaliana***

Methods	Starting year	Advantages	Drawbacks	Refs
Traditional linkage mapping, that is, QTL mapping	1992	• No population structure effect • Identification of rare alleles • Few genetic markers required for a complete genome scan	• Coarse mapping • Limited genetic diversity • Not possible to distinguish between pleiotropic and physically close genes	30
Association mapping with candidate genes	2002	• Fine mapping	• Requires detailed knowledge of the biochemistry and genetics of the trait under study • Approach is biased for previously identified genes	42,146, 147
GWA mapping at the species scale	2005	• Fine mapping (blind approach) • Detection of common alleles	• False positives due to population structure • False negatives after controlling for population structure • Reduced power to detect rare alleles or weak-effect alleles • Genetic and allelic heterogeneity	17,46
Dual linkage–association mapping at the species scale (FIG. 2)	2007	• Fine mapping (blind approach) • Identification of false positives and false negatives	• Phenotyping of several thousands of individuals • Numerous traditional linkage mapping populations required • Genetic and allelic heterogeneity	23,49, 52
GWA mapping in regional mapping populations	2010	• Fine mapping (blind approach) • Diminished population structure effect • Detection of genes involved in local adaptation	• Potential for limited phenotypic variation • Increased linkage disequilibrium: less precise than using a worldwide sample	21,44, 114
NAM at the species scale	Ongoing	• Fine mapping (blind approach) • Identification of false positives and false negatives • High-density genotyping of a small number of founders lines (<30)	• Importance of the crossing schemes and the number of founders • Phenotyping of several thousands of individuals • Genetic and allelic heterogeneity	63,64, 148

GWA, genome-wide association; NAM, nested association mapping; QTL, quantitative trait locus.

Quantitative trait locus
Genomic region containing one or more genes that affect the variation of a quantitative trait.

Genome-wide association
Whole-genome scans that test the association between the genotypes at each locus and a given phenotype.

Seed dormancy
Mechanism that prevents seed germination, even under conditions that promote germination.

Ionomics
The study of the composition of mineral nutrients and trace elements in living organisms.

Genotype–environment interaction
An effect of a locus that changes in magnitude or direction across environments.

Trade-off
Negative genetic and phenotypic correlation between two traits arising from the need of the individual to allocate resources to alternative functions.

will those designed to phenotype plants in only one location, while also offering insights into the genetic bases of genotype–environment interactions (G×E interactions)[24,25]. Last, in nature there are a multitude of selective pressures that simultaneously act on individuals. This should lead to selection for a global phenotypic optimum that results from trade-offs among specific traits[26]. We argue that the adaptive value of a specific trait is best understood in the context of other phenotypic traits, when its relative contribution to fitness is known.

Next-generation sequencing (NGS) technologies[27–29] will additionally facilitate access to the causal polymorphisms that underlie natural variation of complex traits. This is clearly an exciting time to map the genetic bases of complex traits in *A. thaliana* and put them in the context of ecology and adaptation in nature. In this Review, we first assess alternative methods for identifying natural alleles that control quantitative traits, addressing them chronologically according to their use in *A. thaliana* (TABLE 1). We then outline the prospects for introducing ecological approaches to the genetic analyses.

Traditional linkage mapping

Based on a genetic map, traditional linkage mapping (also known as QTL mapping) in *A. thaliana* refers to the use of experimental populations (FIG. 1; TABLE 2) ranging from classical F2 populations[30] to the more recently developed multiparent advanced generation intercross (MAGIC) lines[31]. Recombinant inbred lines (RILs) remain the most popular experimental populations in *A. thaliana*: as these populations are almost completely homozygous, they allow one to replicate genotypes within an experiment and/or among several environmental conditions. More than 60 such RIL families have already been developed. RILs typically define QTL regions of a few megabases covering thousands of genes[32,33], although the resolution can be as high as 300 kb (~50 genes) for the MAGIC lines[31]. A major drawback of this approach, therefore, is that the resultant mapping is coarse (TABLES 1,2). Three options can be envisaged to resolve this issue. First, after a QTL has been localized to a relatively narrow region (3 cM or less, for example (see REF. 34)), fine mapping and cloning of the QTL can be carried out, typically using near isogenic lines (NILs) or heterogeneous inbred families (HIFs)[35,36]. Second, QTL mapping can be complemented with microarrays or sequence prediction for inactivated genes within QTL intervals[37,38]. Third, a promising alternative to fine mapping in QTL regions involves direct sequencing of segregating populations to identify causative mutations, as first demonstrated for induced mutants in *A. thaliana*[39,40] and then implemented for quantitative traits in yeast[41].

Unlike GWA mapping, traditional linkage mapping is useful for identifying rare alleles and is not subject to the effect of population structure (see the later subsection 'GWA studies: the disadvantages'; TABLES 1,2). The genetic bases that are identified by QTL mapping, however, are specific to the parental lines of the experimental segregating populations and may not be representative of the genetic variation on which natural selection acts.

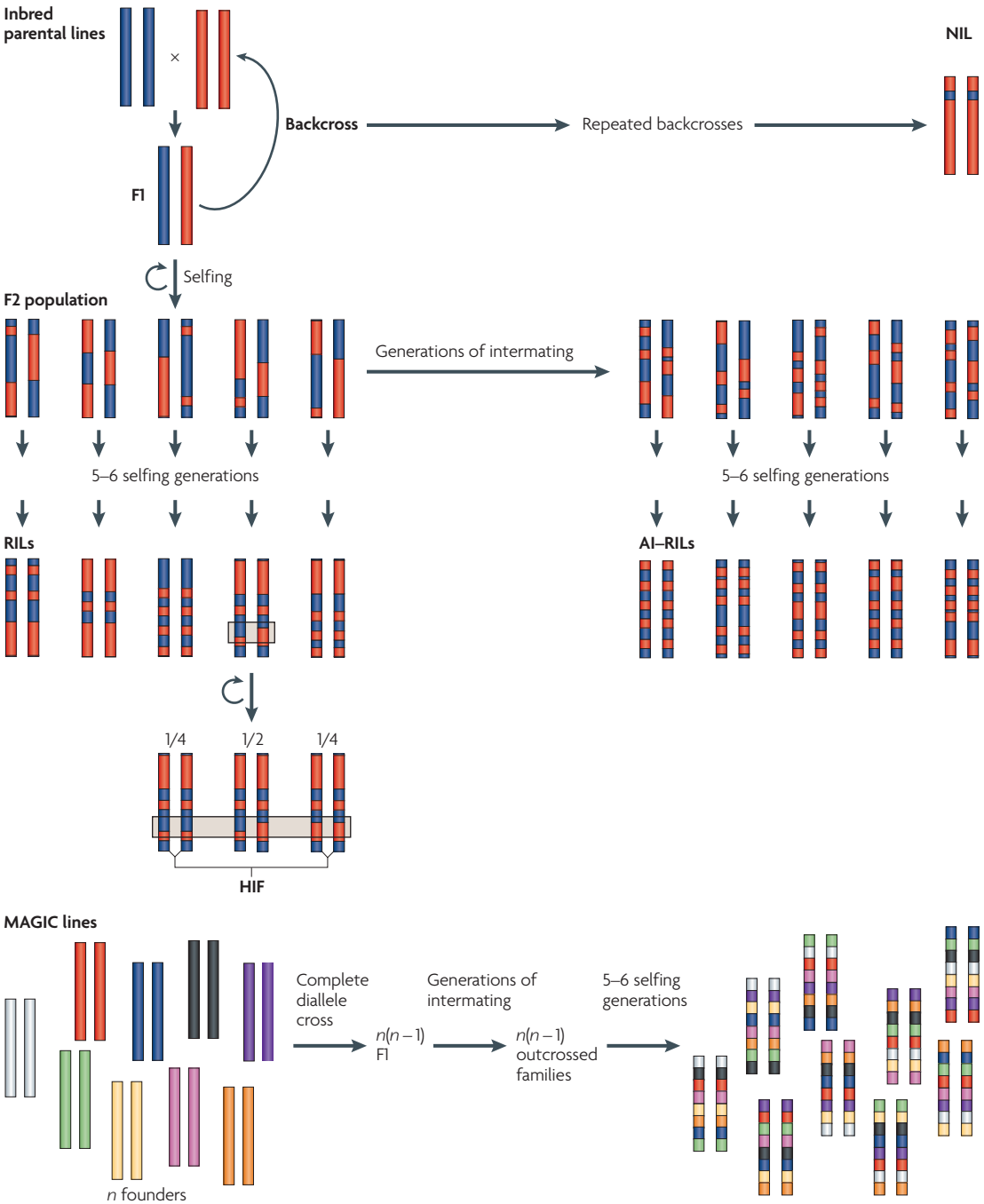


Figure 1 | **Linkage mapping populations in *Arabidopsis thaliana*.** The mapping resolution and the genetic diversity in the linkage mapping populations will depend on the number of founders, generations of intermating and generations of selfing. See TABLE 2 for the advantages and drawbacks of each mapping population. AI-RILs, advanced intercross–recombinant inbred lines; HIF, heterogeneous inbred family; MAGIC lines, multiparent advanced generation intercross lines; NIL, near-isogenic line; RILs, recombinant inbred lines.

Genetic map
Representation of the position of genetic markers relative to each other, with distances between loci expressed in terms of recombination frequency.

Recombinant inbred lines
Quasi-homozygous lines produced from an initial cross between two individuals, followed by six to eight generations of selfing.

Population structure
Differentiation in allele frequencies among multiple populations.

Linkage disequilibrium
Nonrandom allelic association such that two alleles at two or more loci are more or less frequently associated than predicted by their individual frequencies.

Association mapping, GWA studies and NAM

Association mapping appeared next as an alternative for fine-mapping genomic regions associated with phenotypic variation; this method has taken both candidate gene and genome-wide approaches. The candidate gene approach is especially useful in non-model plant species; however, it requires detailed knowledge of the biochemistry and genetics of a trait[7], making it difficult to apply even in a well-studied species such as *A. thaliana*[42]. By contrast, the genome-wide strategy allows one to search blindly for genomic regions that are associated with a trait of interest[43]. GWA mapping uses natural linkage disequilibrium (LD) to identify polymorphisms that are associated with phenotypic variation. As GWA studies take advantage of recombination events that have accumulated over thousands of generations[9,18], the resolution

Table 2 | **Advantages and drawbacks of linkage mapping populations in *Arabidopsis thaliana***

Mapping material	Advantages	Drawbacks	Time (generations)	Refs
Backcross	• Detecting genetic basis of heterosis*	• Low mapping resolution • Limited genetic diversity	2	149,150
F2 population	• Estimation of QTL dominance	• Genotyping individuals for each phenotyping experiment • Limited genetic diversity	2	52,151
RILs	• Genotyped once • Unlimited replicates	• Limited genetic diversity	7–8	33,58
AI-RILs	• High-resolution mapping • Genotyped once • Unlimited replicates	• Limited genetic diversity	10	152,153
MAGIC lines	• High-resolution mapping (up to 300 kb) • Increased genetic diversity • Genotyped once • Unlimited replicates	• Genetic and allelic heterogeneity	10	31
NILs	• Single introgression segment in homogeneous genetic background • Increased power to detect small-effect QTL • Unlimited replicates	• Time consuming: size of the introgression segment will depend on the number of backcross generations • Limited genetic diversity	>6	36
HIFs	• Single introgression segment in heterogeneous genetic background • Increased power to detect small-effect QTLs • Increased power to detect epistasis • Unlimited replicates • The same genomic region covered by independent HIFs	• Limited genetic diversity	9–10	35,154

*Heterosis is the equivalent to hybrid vigour; superiority in one or more phenotypes of the hybrid individual over the parents. AI-RILs, advanced intercross–recombinant inbred lines; HIF, heterogeneous inbred family; MAGIC lines, multiparent advanced generation intercross lines; NIL, near-isogenic line; QTL, quantitative trait locus; RILs, recombinant inbred lines.

to fine map can be greatly enhanced relative to RILs. *A. thaliana,* in particular, has LD that extends for roughly 10 kb[44], which is a nearly ideal distance for mapping — that is, it extends up to the gene level, so there is no need to develop extensive SNP-tiling arrays.

GWA studies: the advantages. A meta-analysis of GWA studies for 107 phenotypic traits in *A. thaliana* was recently carried out[17], and all of the genetic and phenotypic resources are publically available. In the study, 76 to 194 accessions, that is, genetic lines sampled in natural populations, were phenotyped for traits related to flowering time, developmental characteristics, biotic resistance and/or ionomics. These accessions, which are propagated as homozygous lines, have been genotyped using AtSNPtile1 (REFS 44,45), a custom Affymetrix SNP chip containing almost 250,000 known non-singleton SNPs. The ability of GWA mapping in *A. thaliana* to identify the genetic basis of various phenotypic traits has been demonstrated in three main ways. First, GWA mapping successfully identified resistance genes that were already validated as those underlying resistance to pathogens in *A. thaliana*[46]. For example, the *RESISTANT TO PSEUDOMONAS SYRINGAE 5* (*RPS5*) *R* gene — which is known to recognize the *avrPphB* avirulence gene in the bacterial pathogen strain *Pseudomonas syringae*: Pst DC3000 (REF. 47) — was detected as a single peak of association. The same is true for other known *R* genes, such as *RESISTANCE TO P. SYRINGAE PV MACULICOLA 1* (*RPM1*)[48]. Similarly, association peaks related to qualitative resistance to the downy mildew agent *Hyaloperenospora arabidopsidis* ex *parasitica* (*Hpa*) overlapped with known RPP (resistance to *Hpa*) loci[49]. Second, the main association peak identified by GWA mapping for leaf necrosis in a set of 96 accessions was located within the ACCELERATED CELL DEATH 6 (ACD6) locus, which was functionally validated as the main determinant of natural variation for premature leaf death[50]. Third, based on previous knowledge of the very detailed genetic network of flowering time, enrichment of *a priori* candidate genes has been found for several traits related to floral transition[17,23]. Although this enrichment of *a priori* candidate genes is encouraging, only functional validation will prove that the genes related to flowering transition that were identified under association peaks are true positives.

GWA mapping in humans generally requires thousands of genotyped individuals to account for a small fraction of the genetic variation of complex traits[51]. Even with fewer than 200 genotyped accessions, strong associations have been found in *A. thaliana*, suggesting the occurrence of common alleles of major effect at the species scale[17]. As GWA mapping is a blind approach, it also facilitates the identification of new regions containing no *a priori* candidate genes[17,23], potentially enhancing our knowledge of genetic networks related to complex traits.

GWA studies: the disadvantages. GWA mapping in *A. thaliana* suffers from two major limitations. First is the problem of false positives due to population structure (FIG. 2). Population structure may be a problem that is especially great when both phenotypic and

SNP-tiling array
A microarray platform combining SNP genotyping and whole-genome tiling; it contains probes for each allele and each strand of several thousands of SNPs.

Non-singleton SNP
A SNP polymorphism that is present in at least two individuals.

genetic differentiation vary with geographic distance[52]. Statistical methods to control for population structure can reduce the inflation of false-positive associations (see the 'Statistical analyses for GWA mapping' subsection) but may also introduce false negatives (rarely considered in GWA studies in humans); that is, causative genetic markers may be lost when applying GWA methods that control for population structure (FIG. 2). One potential solution is to carry out GWA mapping on a less structured sample of accessions; however, this alternative is not feasible when the phenotypic variation occurs on the scale of the species[21]. In such cases, a combination of traditional linkage mapping and GWA mapping may be a better alternative for reducing the rate of false positives[49,53] and for detecting false negatives[52] (FIG. 2). Dual linkage and association mapping was recently shown to outperform each method in isolation when applied to flowering time data for *A. thaliana* grown in the field[23]. For the 50 best-associated SNPs, the enrichment ratio in *a priori* candidate genes almost doubled when considering candidate genes overlapped by QTLs detected using RILs relative to candidate genes only (7.4 versus 4.1, respectively). This dual mapping strategy estimated that GWA analysis alone led to a false-positive rate of 40% and a false-negative rate of 24%.

Second, genetic heterogeneity and/or allelic heterogeneity may interfere with the detection of SNPs linked to phenotypic variation (FIG. 3). It is well known that different combinations of genes can lead to the same phenotype[54]. For example, the genetic bases of the coat colour that confers a selective advantage of crypsis[55] in the coastal beach mouse *Peromyscus polionotus* differs between populations in the Mexican gulf and on the east coast of Florida[56]. In several plant species, different QTLs[23,57,58] and/or different alleles at the same QTL[59–62] are responsible for an early-flowering trait. As a first step to control the effects of genetic and allelic heterogeneity in *A. thaliana*, >1,100 *A. thaliana* lines have been collected and genotyped using the Affymetrix 250K SNP-tiling array, AtSNPtile1. This set, called the RegMap lines, covers much of the geographical range of the species but with particularly strong representation of accessions from Sweden, the United Kingdom and France (J.B., J. Borevitz and M. Nordborg, unpublished data). The comparison of GWA mapping results among subsets of this collection will reveal the extent of genetic and allelic heterogeneity in *A. thaliana*. Although they capture much of the genetic variation in the species, the RegMap lines are nonetheless geographically limited, and more extensive sampling in additional geographic regions would be desirable.

Nested association mapping. NAM, which was originally developed in *Zea mays*[62,63], is a promising method that is currently under development for fine-mapping QTLs in *A. thaliana*. NAM takes advantage of both historic and recent recombination events to combine the advantages of traditional linkage mapping (that is, low marker-density requirements and high allele richness) and association mapping (that is, high mapping resolution and high statistical power), while being less susceptible to false positives and false negatives[64]. The 250K SNP genotyping of many *A. thaliana* accessions that serve as parents in RIL populations or MAGIC lines will soon enable the NAM strategy to be undertaken. In practice, this will involve projecting the genetic information from parental lines onto the experimental populations used in a traditional linkage mapping study. The joint analysis of data sets from natural accessions and NAM populations should greatly increase our power to finely map genomic regions associated with phenotypic variation, although statistical analyses adapted to the hierarchical design of NAM remain to be developed.

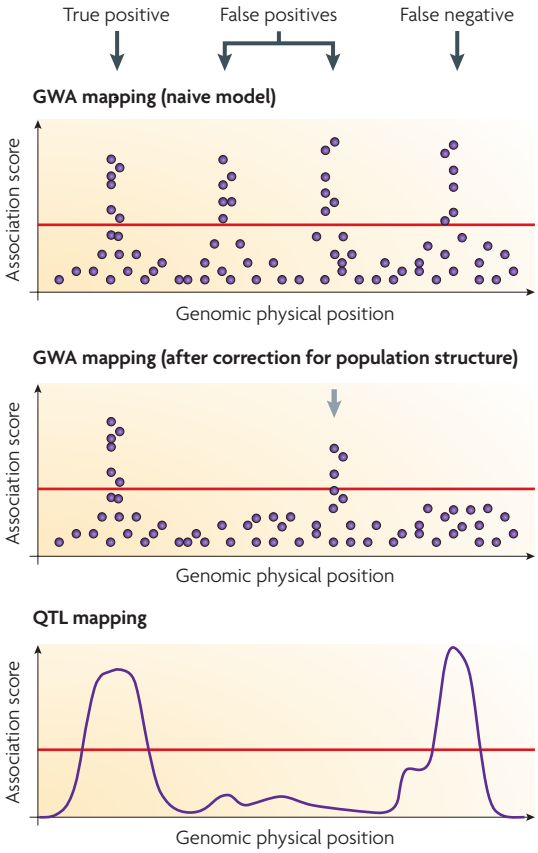


Figure 2 | **Advantages of combining association and traditional linkage mapping methods.** Dual linkage–association mapping allows true positives and false negatives to be distinguished from false positives. True positives are causative SNPs that have been detected by genome-wide association (GWA) mapping and are overlapped by quantitative trait locus (QTL) regions. Population structure corrections highlight false positives that correspond to false phenotype–genotype associations. Because statistical methods that control for population structure only reduce (but do not abolish) the inflation of false positives, false positives may remain (grey arrow). In such cases, the remaining false positives are not validated by QTL regions, demonstrating the added value of QTL mapping in the detection of true positives. False negatives are causative SNPs that are lost as an artefact of population structure corrections but can be validated by QTL regions. The horizontal red line indicates the significance threshold for a phenotype–genotype association.

Genetic heterogeneity
The same phenotypic value caused by different mutations at different genes.

Allelic heterogeneity
The same phenotypic value caused by different mutations at the same gene.

Crypsis
Capacity of an organism to avoid detection by other organisms by blending into the environment.

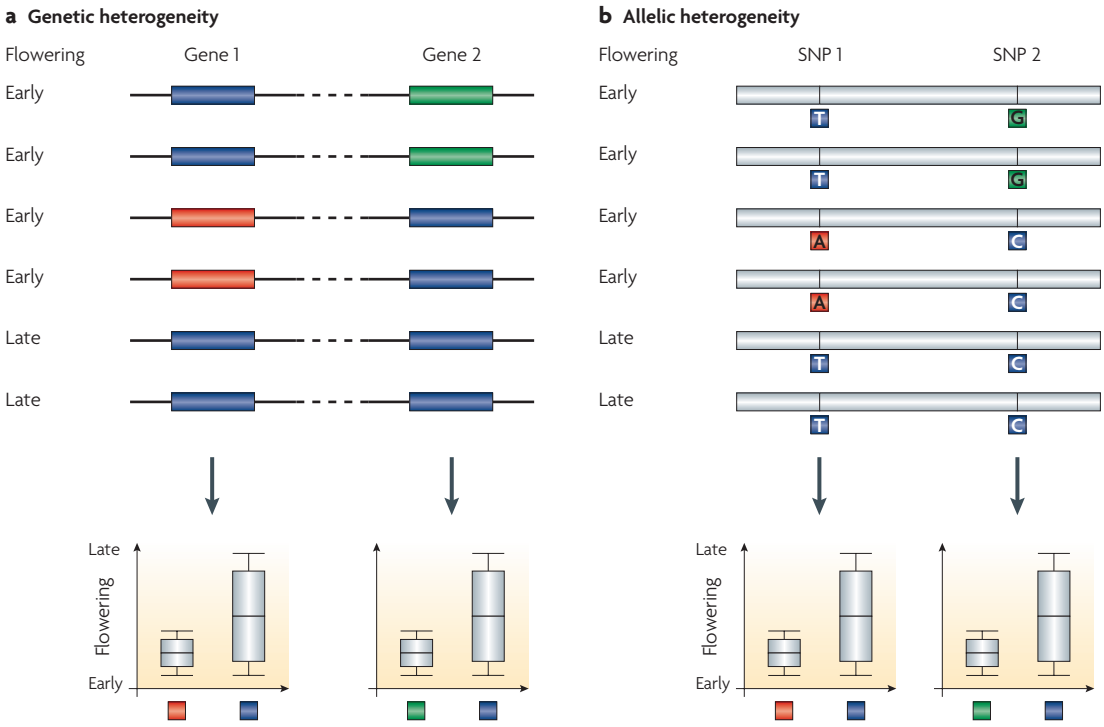


Figure 3 | **Genetic and allelic heterogeneity. a** | Genetic heterogeneity. When alternative genes lead to the same phenotype, genetic heterogeneity can impede detection of the genes that underlie natural phenotypic variation. Here, early flowering occurs through different quantitative trait loci (QTLs), that is, genes 1 (red allele) and 2 (green allele). **b** | Allelic heterogeneity. Two alleles at the same gene (T→A and C→G mutations) may confer a similar phenotype, such as early flowering. Box plots associated with genetic and allelic heterogeneity are represented in the lower panel for each polymorphic gene and polymorphic allele, respectively.

GWA tools: marker types. SNP markers are increasingly popular for mapping because of their high frequency in the genome[29]. SNP-tiling arrays (AtSNPtile1) containing probe sets for 248,584 SNPs have been designed using the complete genome sequences of 20 natural accessions[44] that represent the maximal genetic diversity among a set of 95 worldwide accessions[65]. Given the relatively small genome size of *A. thaliana*, this Affymetrix genotyping array provides, on average, one SNP every 500 bp[44], which is more than adequate coverage for GWA mapping. Indeed, even though LD extends an average of 10 kb among worldwide accessions of *A. thaliana*, the coverage afforded by this array is sufficient to accommodate high variability in LD across the genome[44]. For example, LD is less extensive around loci that have experienced balancing selection for long evolutionary times, such as those encoding pathogen resistances[66–68].

SNP markers represent only a fraction of the available genetic polymorphisms. The ongoing 1001 Genomes sequencing project will permit access to most DNA polymorphisms in *A. thaliana*[69], including structural variants such as copy number variation (CNV) or insertions-deletions (indels). Indels contribute to phenotypic variation in *A. thaliana* for traits such as flowering time[59,60,70] and resistance to herbivory and pathogens[67,71]. Initial analysis of ~200 German and Swedish whole-genome sequences revealed 5 million SNPs (M. Nordborg, personal communication). Information on the full genome should facilitate direct access to causal variations, greatly facilitating a mechanistic understanding of functional polymorphisms. Although it might seem that consideration of whole-genome sequences would vastly increase the computational time required for testing associations, many SNPs will be linked, and this should enable statistical analyses to be hierarchical based on a limited set of SNPs.

Epigenetics is also well known to shape phenotypic variation and should be considered in efforts to understand the evolution of complex traits[72,73]. In a genome-wide survey of loci on chromosome 4, DNA methylation was found to be highly polymorphic among a set of 96 natural accessions of *A. thaliana*[74]. Epigenetic variation can account for up to 30% of the variation in flowering time and plant height in *A. thaliana*[75]. Given the recent technical revolution, epigenome characterization at single-base-pair resolution can be envisioned[76–78]. Indeed, genome-wide scans of DNA methylation are underway in natural accessions of *A. thaliana*. The combination of genotypic and epigenetic information will help to tease apart the effect of DNA sequence variants from that of DNA methylation variants. The development of epigenetic RILs (epiRILs)[75] will also enable the combination of both linkage and association mapping at the DNA methylation level.

Balancing selection
Evolutionary processes that maintain genetic diversity within a population for longer than expected under neutrality. Processes include heterozygote advantage, frequency-dependent selection and variation of fitness in space and time.

Epigenetic RILs
Quasi-homozygous lines that are almost identical at the genetic level but segregate at the DNA methylation level. EpiRILs are produced from an initial cross between two individuals with few DNA sequence differences but contrasting DNA methylation profiles, followed by six to eight generations of selfing.

Given the ever-increasing genetic and epigenetic information for each *A. thaliana* accession, GWA studies will soon suffer from the problem of large dimensionality of polymorphisms. Because the inclusion of additional information may generate more false-positive associations between phenotype and polymorphic markers, statistical tools to appropriately reduce the false-positive rate are in demand.

Statistical analyses for GWA mapping. The effect of SNPs on phenotypes can be tested by using one of several models: non-parametric methods, such as the Wilcoxon rank-sum test for ordered categorical and quantitative phenotypes, or Fisher's Exact Test for binary phenotypes. However, these are relatively naive models because they fail to take into account the confounding effects of population structure. Numerous methods have been developed to account for confounding due to population structure (reviewed in REF. 79). The EMMA[80] software includes a matrix of genotype similarity among the accessions in its mixed linear model (MLM); this matrix has been shown to efficiently correct for the effects of population structure in *A. thaliana*[17], suggesting that the structure of the kinship matrix may well represent both population structure and cryptic relatedness[81]. One limitation of the MLM might be the dependence of associations on minor allele frequencies: strong phenotypic associations are more readily detected when the minor allele frequency is low[17]. Although rare alleles may, at times, be associated with strong phenotypic effects[82], much of this enrichment is likely to be spurious[17].

Given the increasing number of individuals genotyped (with increasing numbers of markers), various methods have been developed to reduce computing time while maintaining or improving statistical power to control for population structure and cryptic relatedness. Such methods include 'compressed MLM' and 'population parameters previously determined (P3D)'[83] — both of which are implemented in the software TASSEL[84] — and a similar variance component approach that is implemented in the EMMAX software[85]. These methods involve first estimating the contribution of population structure to the phenotype using a variance decomposition model. The resulting genetic variance and residual variance values are then kept fixed in a model that tests an association between each marker and the phenotype.

Many phenotypic traits might be structured as a network, time series or hierarchy (BOX 1). Numerous GWA mapping extensions are underway to take advantage of such phenotypic structure for increasing the detection of associated genome variations[86]. Because the causal allele for the same phenotype might be different among populations[12,56,87], multi-task regularized regression has also been used to find causal loci in multi-population GWA mapping[88] and to reduce the rate of false positives due to population structure. Such structured association-mapping algorithms are often publically available on platforms such as GenAMap.

Functional validation

Although GWA mapping has greatly enhanced our ability to fine-map the genomic regions that are linked to natural variation, functional validation remains the gold standard for identifying causative polymorphisms. This is facilitated by the impressive genetic resources in *A. thaliana*[16,34,89], such as non-targeted random disruption or alteration (ethyl methanesulfonate (EMS)- and transposon-mediated mutagenesis, T-DNA mutants and unimutant collection) and specific targeted disruption or alteration (gene silencing by amiRNA), which allow quantitative complementation and quantitative knockdown, respectively.

Nevertheless, we must keep in mind that QTLs that are detected by either QTL mapping or GWA mapping will often result from allelic variation that cannot be captured by knockout or knockdown lines. In addition, QTLs that are detected in field experiments typically explain less than 10% of phenotypic variation[23,90]. For both of these reasons, it is important to create isogenic material against which the effects of particular genes can be compared while also controlling for effects of genetic background and chromosomal location. As an example, the use of a Cre–*lox* system facilitated the detection of small (9%) differences in seed production that were associated with the presence or absence of the *RPM1* pathogen-resistance gene under field conditions[91]. Extensions of this technology to allow consideration of allelic series will prove to be useful but have not yet been applied to dissecting QTLs[92].

To date, more than 30 genes involved in natural variation of complex traits have been functionally validated in *A. thaliana*[93]. However, functional validation is still lacking for a range of quantitative traits that are thought to be strongly related to plant fitness, such as the duration of the reproductive period[94] or disease resistance and tolerance to pathogens[95,96]. Also noteworthy is the absence of functional validation of ecologically important genes as scored under field conditions. Because natural selection acts in nature, where the environment and associated cues are complex, we argue that both GWA mapping and functional validation under natural conditions will be crucial for understanding adaptive evolution.

Adding ecology to association mapping

Geographical scale of adaptation. The maintenance of phenotypic diversity and life history evolution will largely depend on the scale of environmental heterogeneity[97,98]. As a consequence, the scale at which GWA mapping should be performed will depend on the scale at which natural variation is observed, which in turn depends on the ecological factors acting as selective pressures. Bolting time, for example, is correlated with latitude in *A. thaliana,* making climatic variables plausible ecological factors acting across the range of the species[99,100]. Other selective pressures, such as attack by natural enemies, soil composition and interspecific competition, may well be heterogeneous among geographically close populations, and even among individuals within a population[101–103]. In addition to geographical variation is the role of temporal variation, which affects the recruitment of adaptive alleles[104,105]. The environmental grain might thus differ among phenotypic traits across spatial and temporal scales[106–108] and clearly needs to be considered in the design of regional or local mapping populations.

Non-parametric methods
Statistical methods, also called distribution free methods, that are not based on a normal distribution of data.

Mixed linear model
Statistical model containing both fixed effects and random effects.

Multi-task regularized regression
Joint association analysis of multiple populations with a multi-population group lasso using L_1/L_2 regression.

T-DNA
Transferred DNA of the tumour-inducing (Ti) plasmid of some bacterial species into the nuclear DNA genome of the host plant.

Unimutant collection
A collection of 31,033 publically available homozygous T-DNA insertion lines in *Arabidopsis thaliana* representing 18,506 individual genes; produced by the Salk Institute.

AmiRNA
Artificial microRNAs that target specific genes for silencing.

Cre–*lox*
Transgenic technology creating isolines with identical genomes, except for the gene of interest. The resulting paired isolines are created by first introducing the gene of interest with a selectable marker into the genome and then excising the gene of interest. Modifications of this approach can be used to create allelic series.

Environmental grain
The scale of temporal and spatial environmental variation that is perceived by an organism.

Projects that aim to describe the environmental grain of diverse selective pressures would be useful, especially if they emphasize factors such as biotic interactions[22], which are poorly studied in natural populations of *A. thaliana* but are well known to influence the evolutionary trajectories of populations in other plant species[109,110]. Soil composition is another important factor that may drive adaptive responses in plants[111,112]. Indeed, a short life cycle emerged as an adaptive response to high concentrations of phosphate, as experimentally validated in *A. thaliana*[113]. Furthermore, natural populations of *A. thaliana* associated with coastal and saline soils in Europe were recently found to be enriched for a weak natural allele of *HIGH-AFFINITY K+ TRANSPORTER 1;1* (*HKT1;1*), which confers elevated salinity tolerance[114].

Box 1 | **Structured phenotypic traits**

Many phenotypic traits can structured as a hierarchy, time series or network.

Hierarchy

Phenotypic variation of trait 1 may be decomposed by variation that is observed in traits 11 and 12, themselves decomposed by variation that is observed in intermediate phenotypes (see the figure, part **a**). For example, the life cycle in annual plants may be decomposed into a vegetative phase and a reproductive phase. The vegetative phase is composed of the time interval between sowing and bolting and the interval between bolting and flowering. The reproductive phase is composed of the flowering period and the seed maturation period. Note that the flowering and maturation periods may overlap.

Time series

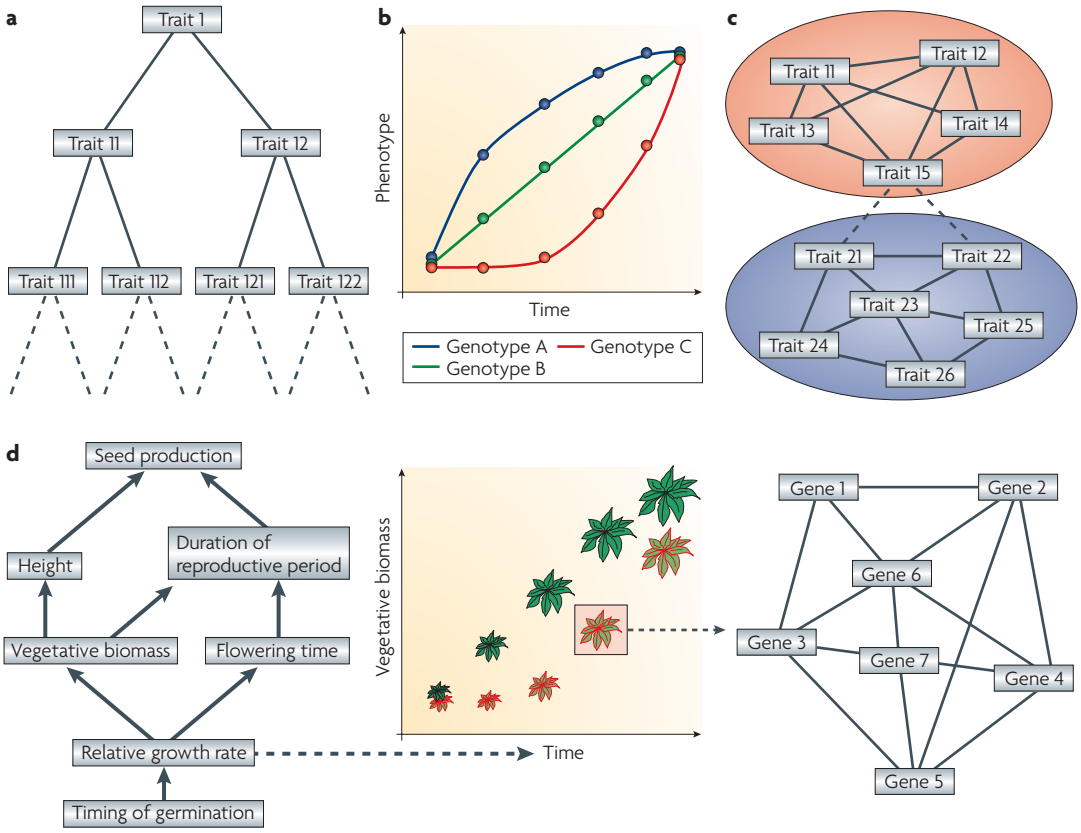
In this case genotypes are measured for the same phenotype at equally spaced, discrete time intervals (see the figure, part **b**). Time series analysis deals with the non-independence of data points taken over time. Examples of time series analysis in *Arabidopsis thaliana* include studies of disease symptoms, aerial biomass growth, root growth and cold tolerance.

Quantitative trait network

Gene expression, primary and secondary metabolite profiles, composition of mineral nutrients and trace elements could be studied as quantitative trait networks. Part **c** shows the connection between two sub-networks, each corresponding to a group of intercorrelated traits.

Interlink among hierarchy, time series and network

Seed production results from a combination of morphological, phenological and life history traits (see the figure, part **d**, left). A component of the hierarchy, relative growth rate, is estimated by scoring the vegetative biomass at successive times (middle). Vegetative biomass estimated at a specific time results from the intercorrelated expression of many genes (right).



A benefit of studying adaptation in plants is that they stand still, and because the collection site of many accessions, including all RegMap lines, is known, it is easy to envision scans for genes associated with particular environmental variables. Several such studies are currently underway and are likely to produce a rich list of candidate genes for ecological testing.

Complex environmental cues. Consistent with observations in other plant species[115], QTL mapping analyses in *A. thaliana* have revealed different QTLs for the same traits measured in greenhouse conditions and in common gardens[23,90,116,117]. The high resolution conferred by GWA mapping in *A. thaliana* strengthens this observation. Only two out of 25 candidate genes associated with flowering time when measured under field conditions have also been proposed as candidate genes for flowering-time phenotypes in GWA mapping studies scored under greenhouse conditions[23]. In a natural setting, plants are exposed to a greater range of day lengths and greater daily fluctuations in temperature, humidity and light quality than are typically encountered in the greenhouse. As a consequence, many circadian clock-related genes entrained by photoperiod and thermocycles have been detected by GWA mapping for flowering time scored in ecologically realistic conditions[23], but not in the greenhouse.

Recent QTL mapping studies of flowering time in *A. thaliana* have attempted to simulate outdoor climatic conditions in growth chambers by varying photoperiod and temperature over time[118–120]. Although this is a good first step, these studies used climatic conditions based on the average across several years and therefore considered much smoother environmental changes than the daily stochastic variation that is observed in outdoor conditions. Similarly, these studies do not take into account biotic interactions such as competition, herbivory and pathogen attacks that may trigger various flowering-time responses[121–123]. The next challenge in identifying genes underlying ecologically relevant traits in *A. thaliana* will certainly be the phenotyping of plants that have established themselves in natural populations without human interference.

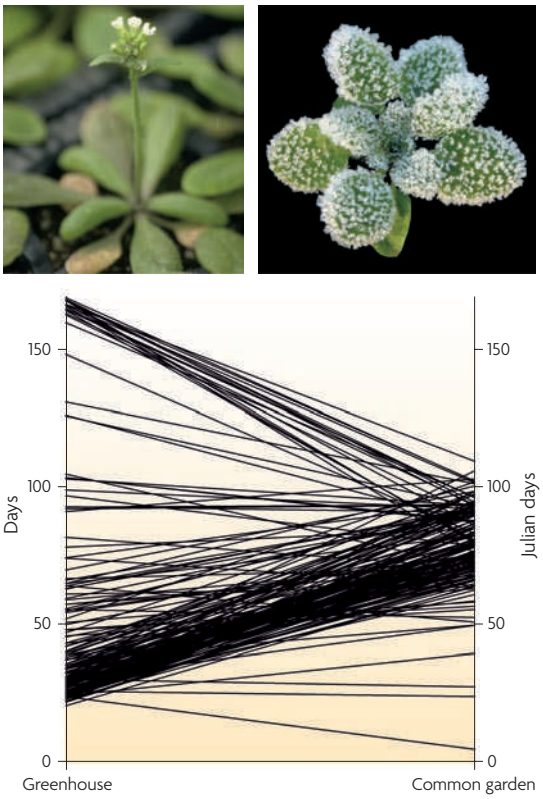


Figure 4 | **Reaction norms of flowering time between the greenhouse and the common garden.** *Arabidopsis thaliana* reveals extensive genotype–environment interactions between greenhouse and outdoor conditions. Flowering time has been scored for 183 worldwide accessions in greenhouse conditions (20 °C, 16 hour photoperiod)[17] and in a common garden at the University of Lille (Northern France)[23]. In the greenhouse, flowering time is expressed in days since sowing. In the common garden, seeds were sown in late September and flowering time is expressed in Julian days since 1 January. Note that most accessions flowered in early spring, that is, late March, in the common garden. Images courtesy of B. Brachi, Université des Sciences et Technologies de Lille.

Genotype–environment interactions. Like many other plant species with a worldwide distribution, *A. thaliana* can be found in contrasting habitats. Phenotypic plasticity might thus be a key factor in the process of adaptation to newly colonized geographical areas[124,125]. The selfing reproductive system of *A. thaliana* enables one to replicate genotypes within and between environments, allowing direct examination of phenotypic plasticity. Such studies have revealed extensive genetic variation for reaction norms, suggesting the occurrence of strong G×E interactions in *A. thaliana*[123,126] (FIG. 4).

Work has begun to dissect the genetic architecture of G×E interactions in *A. thaliana* for various environments, such as seasons, water availability, nitrogen sources and plant density[90,116,122,127–132]. That said, the molecular and mechanistic bases of the functional polymorphisms underlying G×E interactions remain poorly known. A recent and large experiment to phenotype *A. thaliana* mutants that are impaired in particular flowering-time pathways has started to fill this gap by phenotyping plants in common gardens located in different geographical regions[24]. The authors demonstrated that early flowering conferred by loss-of-function alleles at the *FRIGIDA* (*FRI*) gene was negated by a shift of a few days in germination in early autumn. Very recently, 473 *A. thaliana* accessions were phenotyped for flowering time across two planting seasons in each of two simulated local climates (Spain and Sweden) in growth chambers[133]. In this study, all 12 flowering time QTLs detected by GWA mapping showed sensitivity to seasonal planting and/or simulated local climate. Other GWA mapping experiments performed in multiple geographic regions, such as the flowering time studies that constitute the Ecological Genomics of *Arabidopsis*

Phenotypic plasticity
The ability of an organism to develop a phenotypic state, depending on its external and internal environment.

Reaction norm
The set of phenotypes expressed by a genotype under different environmental conditions.

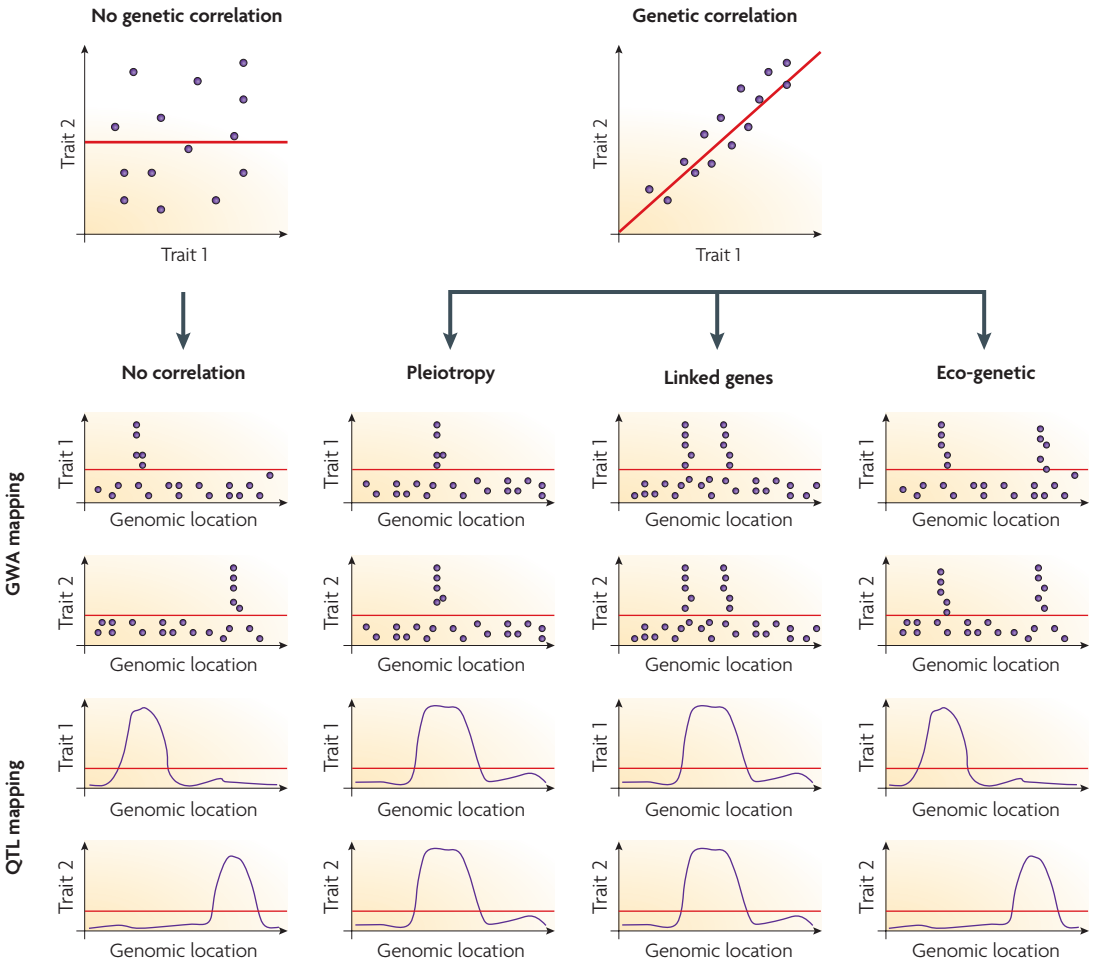


Figure 5 | **Unravelling the origin of genetic correlations.** Both genome-wide association (GWA) and quantitative trait locus (QTL) mapping will reveal distinct genomic locations that are associated with natural variation of two uncorrelated phenotypic traits. Genetic correlations might originate from pleiotropic genes, from physically linked genes or from distinct genes that have been selected by covarying ecological factors. Pleiotropy: the same genomic location is identified by GWA mapping for both traits and is overlapped by the same QTL region. Linked genes: physically close genomic locations are identified by GWA mapping for both traits and are overlapped by only one QTL region. Eco-genetic: physically distant genomic locations are identified by GWA mapping for both traits. Each genomic location is overlapped by only one QTL region.

Development (EGAD) project, are expected to make further significant advances in the understanding of G × E interactions in *A. thaliana*. As extensive year-to-year variation in seed production of the same *A. thaliana* genotype has been detected at one Swedish field station across 8 years[22], GWA mapping experiments will also be usefully replicated across successive years.

Quantitative traits network. Individuals are simultaneously confronted with multiple selective pressures, leading to selection for a global phenotypic optimum that results from trade-offs among specific traits[26,134]. For a selfing annual such as *A. thaliana*, seed production — a proxy of fitness in *A. thaliana* — results from a combination of morphological, physiological, phenological and life history traits (BOX 1). In humans, this corresponds to clinical outcomes that can be thought of as a synthesis of intermediate phenotypes (that is, risk factors)[135]. Path analyses have been used to describe the phenotypic networks that underlie fitness in plants[119,136,137], thereby assessing direct and indirect selection on individual traits. Performing statistical estimation of correlated genome associations[86] may provide insight into the process of adaptation by unravelling the origin of genetic correlations among phenotypic traits, that is, pleiotropy versus genetically linked genes[138].

Still, genetic correlations may also originate from joint selection of covarying ecological factors[139] (FIG. 5). For example, several phenotypic traits in *A. thaliana* are correlated with latitude. Whereas the decrease in solar radiation that is associated with latitude might be thought to select on relative growth rate (RGR)[140], precipitation and/or temperature related to latitude might be the key climatic factors that act on bolting time[100]. In the case of independent genetic bases for correlated traits, crossing two accessions with extreme phenotypes should enable one to break down the genetic correlation observed among accessions (FIG. 5). Thus, whereas

Path analysis
A statistical method that provides estimates of the magnitude and significance of causal relationships between two or more variables.

Pleiotropy
The effect of a gene on more than one phenotypic trait.

GWA mapping will identify the same genomic regions associated with correlated traits, traditional linkage mapping may help to distinguish the origin of genetic correlations.

Conclusion and perspectives

GWA mapping clearly facilitates the identification of genes associated with natural variation in phenotypic traits. In *A. thaliana,* it is also relatively easy to identify false positives and negatives through the strategic combination of traditional linkage and association mapping[23,52], something that is not feasible in humans and many other systems. In addition, the identification of common alleles of major effect suggests a relatively simple genetic architecture for many adaptive traits in *A. thaliana*; such results have not been apparent in maize, mice, flies and humans, in which many loci of small effect have been detected[141]. It remains to be determined whether this is due to the focal species or to focal traits. After functional polymorphisms are validated, it is possible to study the history of selection for these polymorphisms[87,142] and then determine the main contributors to adaptation, that is, new mutations versus standing genetic variation.

Performing ecological genomics by adding ecology to the studies of phenotype–genotype associations will forge a better understanding of adaptation in *A. thaliana*[143], enabling us to retrace the trajectory of adaptive traits in natural populations[2,3] and potentially improve crop yield and quality. Soon, the current revolution in NGS technologies will additionally facilitate ecological genetics in non-model plant species.

Although GWA mapping gives access to the unit of evolution — that is, the gene — the unit of selection — that is, the phenotype — must not be forgotten. Indeed, the next frontier in GWA mapping is high-throughput phenotyping. Due to the development of NGS technologies, genomic resources are rapidly accumulating, but phenotypic data collected in a natural context remain scarce. Automated platforms have been recently developed for phenotyping in growth chambers[144,145], and an International Plant Phenomics Network (IPPN) was recently set up to provide new technologies for high-throughput phenotyping. As genetic variation is exposed to natural selection in nature, such automated platforms are desperately needed to allow phenotyping of plants in natural conditions.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

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